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Mechanisms That Mediate Stem Cell Self-Renewal and Differentiation

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Abstract Stem cells have two common properties: the capacity for self-renewal and the potential to differentiate into one or more specialized cell types. In general, stem cells can be divided into two broad categories: adult (somatic) stem cells and embryonic stem cells. Recent evidence suggested that tumors may contain ''cancer stem cells'' with indefinite potential for self-renewal. In this review, we will focus on the molecular mechanisms regulating embryonic stem cell self-renewal and differentiation, and discuss how these mechanisms may be relevant in cancer cells. J. Cell. Biochem. 103: 709–718, 2008. © 2007 Wiley-Liss, Inc.

Key words: stem cells; embryonic stem cells; self-renewal; pluripotency

Why is news about stem cells so popular internationally, and why are they such an exciting entity to study? At the end of the 20th century, two major breakthroughs in stem cell research were achieved indirectly and directly. First, the successful cloning experiments that led to Dolly in 1997 showed hidden potential that an adult cell nucleus can be reprogrammed to produce an entire animal [Wilmut et al., 1997]. Theoretically the nuclear transfer technology that created Dolly could be used to generate new stem cells, which could be used to treat an individual with his or her own stem cells. Second, the successful derivation of human embryonic stem (ES) cells from blastocysts in 1998 by Thomson and colleagues provides potential cell sources for cell-based therapies for many human diseases [Thomson et al., 1998].

Many diseases are caused by loss of specialized functional cells in organs, that is (i) type I diabetes, in which insulin-producing β cells are destroyed by an autoimmune disorder, (ii) Parkinson's disease, in which dopamine-producing neuronal cells are destroyed, (iii) heart failure, which is caused by the massive loss of cardiomyocytes, and (iv) ischemic diseases, which is primarily caused by endothelial dysfunction.

Stem cell research has expanded in recent years due to the therapeutic potential that is envisioned as a result of progress in this area. In general, stem cells can be divided into two broad categories: adult (somatic) stem cells and ES cells. Research on adult stem cells has recently generated a great deal of excitement; however, the miniscule number of adult stem cells in each tissue and the inability to expand adult stem cells in vitro may limit their usefulness in clinical therapy. The current hope is that successful derivation of human ES cells from blastocysts may provide a cell sources for cell-based therapies [Thomson et al., 1998], because human ES cells are expandable in vitro.

Stem cells are defined as having two properties: (i) the capacity for prolonged self-renewal and (ii) the potential to differentiate into one or more specialized cell types. Adult stem cells are present in many tissues of adult animals and are important in tissue repair and homeostasis. The most studied adult stem cells are hematopoietic stem cells (HSC) in the bone marrow and neuron stem cells (NSC) in the brain. Although adult stem cells were found to be

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more versatile than originally believed [Krause et al., 2001], they differentiate into a relatively limited number of cell types; additionally, some tissues lack stem cells. In contrast, pluripotent ES cells, derived from the inner cell mass (ICM) of the blastocyst, can proliferate indefinitely in vitro, and have the potential to generate every cell type in the body.

To control stem cell self-renewal and differentiation, adult stem cells and ES cells share many important genes and signaling pathways, a property called "stemness" as their stem cell molecular signatures [Ivanova et al., 2002; Ramalho-Santos et al., 2002]. Interestingly, there is recent evidence suggesting indefinite potential for self-renewal in "cancer stem cells" in tumors. These cancer stem cells are distinct from most other tumor cells and they were described as the source of several types of human cancer. Cancer stem cells retain both features of self-renewal and differentiation, but have lost homeostatic mechanisms which maintain normal cell number [Reya et al., 2001]. Understanding of the molecular mechanisms for stem cell self-renewal and differentiation provides a strong foundation not only for stem cell therapy, but also for cancer therapy.

EXTRINSIC AND INTRINSIC REGULATORY NETWORK

The prevailing opinion is that the combination of multiple intrinsic elements and extrinsic signals from microenvironment regulates stem cell behavior. However, some fundamental differences were demonstrated between human ES cells and mouse ES cells. For example, the population-doubling time of human ES cells is significantly longer than that of mouse ES cells. Morphologically, human ES cells form relatively flat and compact colonies. An enzymatic dissociation of human ES cell colonies into single cells can lead to significant decrease of ES cell propagation due to low efficiency of cell attachment, which is not typically associated with mouse ES cells [Sjogren-Jansson et al., 2005]. Undifferentiated hES cells express stage-specific embryonic antigen (SSEA)-3 and SSEA-4 but lack SSEA-1; whereas mouse ES cells express SSEA-1 but lack SSEA-3 and SSEA-4 [Thomson et al., 1998]. Significantly, mouse ES cells remain undifferentiated and proliferate in the presence of leukemia inhibitory factor (LIF) [Williams et al., 1988], whereas fibroblast growth factor-2 (FGF-2), but not LIF, support human ES cells to remain in undifferentiated state [Thomson et al., 1998; Ludwig et al., 2006].

How the extrinsic signals regulate ES cell self-renewal is not well understood. *Oct4*, *Sox2*, and *Nanog* are transcription factors and they play essential roles during early embryonic development (Fig. 1). *Oct4*-deficient mouse embryos fail to develop beyond the blastocyst stage because the ICM cells are not pluripotent, and differentiate into the extraembryonic trophoblast lineage [Nichols et al., 1998]. *Oct4*, a POU domain transcription factor, is expressed

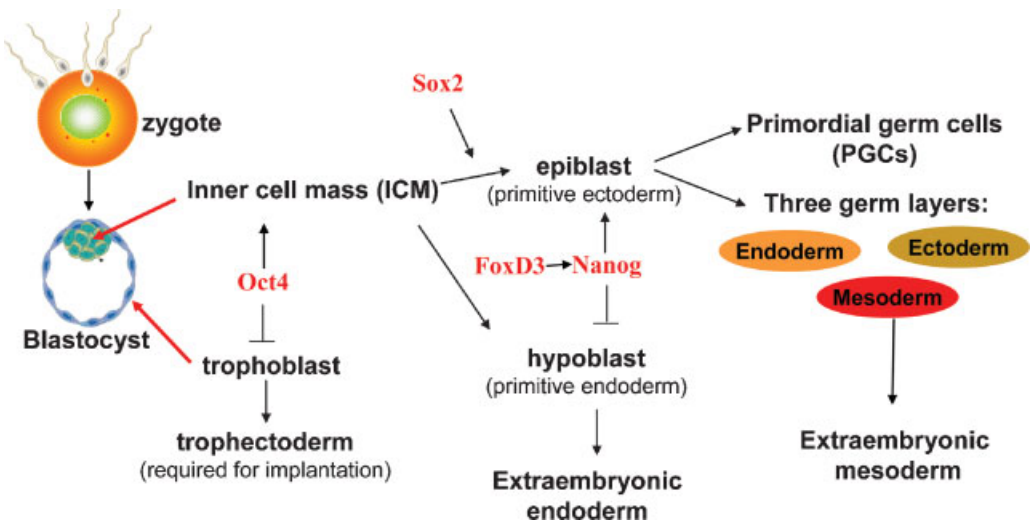

Fig. 1. Transcription factors mediating early embryonic development. Transcription factors, *Oct4, Sox2,* and *Nanog,* play critical roles during early embryonic development. Deficiency of either one of them results in early mouse embryonic lethality, and embryos fail to develop beyond epiblasts.

at high levels in the pluripotent embryonic cells and plays a critical role in the establishment and maintenance of pluripotency of ES cells. Down regulation of *Oct4* promotes differentiation of ES cells in vitro and in vivo [Pesce et al., 1998; Niwa et al., 2000]. *Oct4* can form a heterodimer with *Sox2* that is required for epiblast and extraembryonic ectoderm. Loss of *Sox2* also contributes to extraembryonic endoderm development [Avilion et al., 2003]. *Oct4* and *Sox2* form a complex, and also bind to the *Nanog* promotor region to regulate *Nanog* expression [Kuroda et al., 2005; Rodda et al., 2005]. Removal of *Nanog* results in primitive endoderm differentiation, and ICM fails to generate epiblast [Chambers et al., 2003; Mitsui et al., 2003]. *Nanog*-deficient ES cells lost pluripotency, and overexpressions of *Nanog* induce clonal expansion of mouse ES cells and maintain Oct4 levels independent on Stat3 signal [Chambers et al., 2003; Mitsui et al., 2003]. These studies suggested a central role for *Nanog* in regulation of ES self-renewal and differentiation.

Although extrinsic signals are different in human and mouse ES cells, they eventually lead to regulation of a network of stemness genes. Among the stemness genes, *Oct4*, *Sox2*, and *Nanog* are central transcription factors that maintain the self-renewal and pluripotency of both human and mouse ES cells (Fig. 2) [Pesce and Scholer, 2001; Avilion et al., 2003; Chambers et al., 2003; Mitsui et al., 2003; Boyer et al., 2005; Hyslop et al., 2005; Wang et al., 2006].

The most significant extrinsic factor for mouse ES cells is LIF. The propagation of mouse ES cells is dependent on LIF, which is provided by fibroblast feeders or recombinant protein. It is unclear how LIF acts on the transcription network of *Oct4*, *Sox2*, and *Nanog*. Upon LIF binding, LIF receptor and gp130 form a complex, which activates JAK tyrosine kinases, resulting in phosphorylation STAT transcription factors. Recruitment and activation of STAT3 is essential for self-renewal of ES cells. Blocking the JAK or gp130 C-terminal truncated mutation in mouse ES cells decrease STAT3 activity, and results in ES cell differentiation [Ernst et al., 1999; Niwa et al., 1998]. STAT3 activation is not only necessary but might be sufficient to maintain the undifferentiated state of ES cells in the presence of serum [Matsuda et al., 1999]. Activation of LIFR-gp130 by LIF also activate extracellular-signal-related kinase (ERK) and phosphatidylinositol-3 kinase (PI3K) pathways to maintain mouse ES cells in an undifferentiated state [Burdon et al., 2002]. An in vitro study suggested that STAT3 might bind to an enhancer element of *Nanog* 5′ promoter region, and

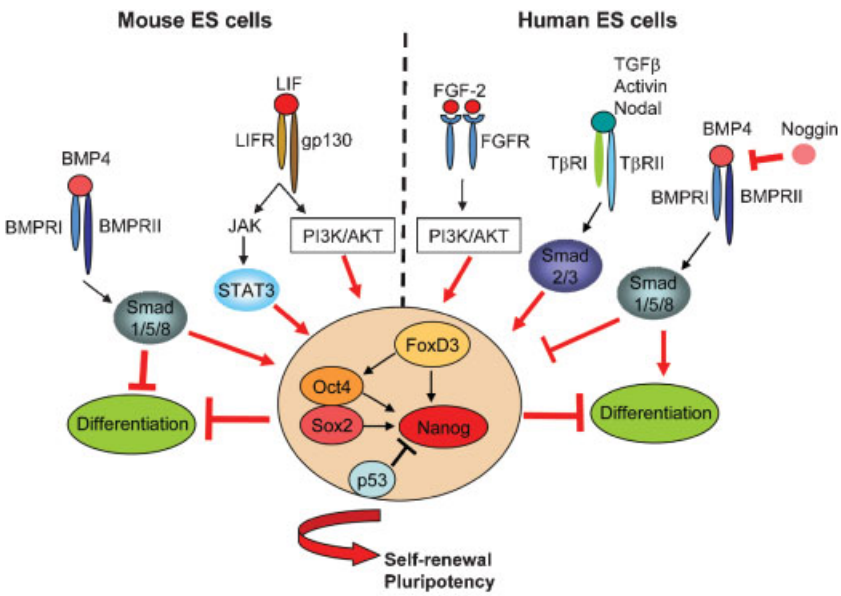

Fig. 2. Extrinsic and intrinsic regulation of ES cell self-renewal and differentiation. *Oct4, Sox2,* and *Nanog* are master genes causing formation of the core transcription regulatory network and control ES cell pluripotency. The activity of the core regulatory network is modulated by multiple extrinsic factors, which are different for human ES cells and mouse ES cells.

activate Nanog transcription [Suzuki et al., 2006]. Nanog expression is also regulated by *FoxD3*, a forkhead family transcription factor highly expressed in ES cells, because *Nanog* 5′ promoter region contains a FoxD3 binding site [Sutton et al., 1996; Pan et al., 2006]. Study by Lin T et al. demonstrated that p53 can suppress Nanog transcription though binding Nanog promoter, and then induce the differentiation of DNA-damaged ES cells, in order to eliminate DNA-damage ES cells [Lin et al., 2005].

LIF is required, but not sufficient, for pluripotent mouse ES cell expansion in vitro in the absence of serum or a feeder cell layer. At least one other extrinsic factor, bone morphogenetic protein 4 (BMP4) is critical to maintain mouse ES cell pluripotency. Both BMP4 and LIF are required to maintain ES cell pluripotency in the absence of serum. When acting individually, LIF induces neuronal differentiation, whereas BMP4 induces mesoderm differentiation [Ying et al., 2003a]. Binding of BMP4–BMP receptors activates Smad1/5/8, which forms a heteromeric complex with Smad4 and translocates to the nucleus. Activation of BMP signaling results in the expression of inhibitor of differentiation (Id) proteins which block lineage commitment and allow self-renewal of ES cells [Norton, 2000; Ruzinova and Benezra, 2003; Ying et al., 2003b]. Inhibition of neuronal differentiation is possibly caused by BMPs blocking the ERK/PI3K signaling cascade [Qi et al., 2004]. Interestingly, under serum-free conditions, overexpression of Bcl-2, an anti-apoptotic protein, allows mouse ES cell expansion in an undifferentiated state in the presence of LIF, even in the absence of bone morphogenic proteins [Yamane et al., 2005]. Overall, signaling cascades involved in cell survival, proliferation, and blockage of commitment function work together for ES cell self-renewal and differentiation.

Oct-4, *Sox2*, and *Nanog* are also the core transcriptional regulatory circuitry of human ES cell pluripotency and self-renewal [Boyer et al., 2005]. However, in human ES cells, although LIF can induce STAT3 phosphorylation and nuclear translocation, it cannot sufficiently maintain the pluripotent status of human ES cells (Daheron et al., 2004, Stem Cells). FGF-2 is an essential component for the maintenance of human ES cells in vitro [Thomson et al., 1998; Xu et al., 2005; Ludwig et al., 2006]. In contrast to mouse ES cells, BMP signaling promotes rapid down-regulation of Nanog and Oct4 in human ES cells, resulting in human ES cell differentiation [Xu et al., 2002]. Activation of FGF signaling and inhibition of BMP signaling cooperatively facilitate long-term maintenance of human ES cells in the pluripotent state [Wang et al., 2005; Xu et al., 2005]. BMPs belong to the transforming growth factor β (TGFβ) superfamily and activate Smad 1/5/8 transcription factors. Other family members, TGFβ, Activin, and Nodal activate Smad 2/3, and promote human ES cell self-renewal and pluripotency [Beattie et al., 2005; James et al., 2005; Valdimarsdottir and Mummery, 2005; Xiao et al., 2006]. Recent studies demonstrate that FGF-2 and TGFβ signalings cooperatively regulate human ES cell pluripotency [Vallier et al., 2005; Greber et al., 2007].

CELL CYCLE REGULATION

An important feature of stem cells is their self-renewal. Appropriate stem cell determination depends on the balance between cell cycle entry and lineage commitment. Adult stem cells are relatively quiescent, which is necessary to maintain tissue homeostasis and reserve for regeneration in response to tissue damages. For example, the majority of hematopoietic stem cells (HSC) at homeostasis remain quiescent, which is regulated by cyclin-dependent kinase inhibitors (CKIs). The absence of $p21^{cip1/waf1}$, the G1 checkpoint regulator, leads HSCs to enter cell cycle, resulting in stem cell exhaustion [Cheng et al., 2000].

Interestingly, another member of CKIs, $p18^{INK4C}$, might have the opposite effect of $p21^{cip1/waf1}$. Deletion of $p18^{INK4C}$ increases self-renewal of HSC, and rescues hematopoietic exhaustion caused by p21 deficiency [Yu et al., 2006]. In contrast to quiescence of adult stem cells, ES cells have proliferative ability and can be prolonged in cultures with an unique cell cycle feature: an abbreviated cell cycle (human ES cells: ~15–16 h; mouse ES cells: ~10 h) [Stead et al., 2002; Becker et al., 2006]. The abbreviated cell cycle of ES cells is due to a short G1 phase. Because the G1 phase is vulnerable to differentiation reagents, such as retinoic acid (RA) [Lukaszewicz et al., 2005], the short G1 phase may contribute to ES cell self-renewal. ES cells express low levels of D-type cyclins (D1, D2, and D3), which are important for G1 phase progression. The expression of cyclin Ds in ES cells in significantly increased during in vitro

and in vivo differentiation [Savatier et al., 1996; Burdon et al., 2002; Jirmanova et al., 2002]. A recent study demonstrated that the progression of G1/S phase of human ES cells was regulated through histone gene regulator p220NPAT and chromatin assembly [Ghule et al., 2007].

CHROMATIN REMODELING

When an adult human somatic nucleus is transferred into Xenopus oocytes, Oct4 expression is induced and differentiation markers are extinguished in the somatic nucleus, suggesting that an adult nucleus can be reprogrammed within embryonic environment [Byrne et al., 2003]. The extrinsic signals that modulate gene expression of intrinsic factors may possibly occur at the chromatin level. Epigenetic modification of chromatin, which is the basic regulatory unit of the eukaryotic genetic material, includes covalent histone modification, DNA methylation, and localization of chromatin to specific nuclear domains. Changing the organization of nucleosomes is the most common way of chromatin remodeling, which needs ATP-dependent chromatin remodeling complexes. Xi and Xie [2005] observed that Imitation SWI (ISWI), an ATP-dependent chromatin remodeling factor in drosophila, regulates germline stem cell (GSC) self-renewal in the drosophila ovary in response to BMP signals from the stem cell niche. The structure change of chromatin likely allows the transcription apparatus to gain access to certain promoters, and then control gene expression and cell fate determination.

DNA methylation and demethylation at CpG islands of genes play an important role in epigenetic modification. DNA methylation patterns, established during embryonic development, are the results of demethylation, de novo methylation and the maintenance of existing methylation. During preimplantation development, both paternal and maternal genomes undergo a wave of demethylation to erase most of the methylation patterns inherited from the gametes. Shortly after implantation, the embryo undergoes a wave of de novo methylation, which establishes a genome-wide hypermethylation pattern [Li, 2002]. Dnmt3b, a DNA methyltransferase, functions in early embryogenesis, especially in ICM, epiblast, and embryonic ectoderm. Both Dnmt3a and Dnmt3b are essential for the stable inheritance or maintenance of DNA methylation patterns in mouse ES cells [Chen et al., 2003]. For example, demethylation of the *Oct4* promoter is critical for its expression in order to keep ES cell in a pluripotent state [Simonsson and Gurdon, 2004]. Deficiency of methyltransferases, either DnmtI or both Dnmt3a and Dnmt3b, results in loss of ES cell pluripotency [Jackson et al., 2004].

Histone modification, such as methylation and acetylation, also plays an important role in changing chromatin structure. Some histone modifications result in transcriptional activation, whereas others lead to gene repression. McCool et al. [2007] observed a global increase in acetylation in histone H3 and H4 during early differentiation of mouse ES cells induced by LIF withdrawal, suggesting that histone modification participates in the loss of the undifferentiated state of ES cells. However, modification of histone acetylation alone is not sufficient for irreversible differentiation. The Polycomb Group (PcG) proteins, transcriptional repressors, are chromatin modification factors, and regulate ES cell self-renewal and pluripotency by repressing a large number of developmental regulators in murine and human ES cells [Boyer et al., 2006; Lee et al., 2006]. Many PcG target genes are repressed by PcG repressive complexes PRC1 and PRC2 to maintain pluripotency in ES cells. Deficient of either the PRC2 members, *Ezh2* or *Eed*, or the PRC1 component *Rnf2* leads to the defects of early embryonic development and of ES cell pluripotency, suggesting that PcGs are critical in maintenance of ES cells [Sparmann and van Lohuizen, 2006].

SELF-RENEWAL OF ES CELLS AND CANCER

The central question about cancer is how do normal cells mutate? Because ES cells resemble cancer cells in many ways, especially in their ability to growth indefinitely, newly arising cancer cells may share some of the same signaling pathways as those normally used in ES cells for self-renewal. It is reasonable to propose that some embryonic genes may be re-expressed or re-activated in cancer cells. For example, Oct 4 and three additional embryonic genes are expressed in human tumors, but not in normal somatic tissues [Monk and Holding, 2001]. Recently, Wang et al. [2003] demonstrated that Oct4 was also expressed in human breast cancer cell lines and all human primary breast

carcinomas examined, but not in normal human breast tissue. These studies suggest a link between Oct4 and tumorgenesis. Genes specifically expressed in human embryonic stem cells, but not expressed in somatic cells may have greater potential as targets in cancer treatment.

Evidence shows that many pathways that are classically associated with cancer also regulate normal ES cells. For example, the Wnt-β-catenin pathway, which directs cell fates during embryonic development, contributes to a variety of human cancers when it is deregulated [Peifer and Polakis, 2000]. When cells are exposed to Wnt proteins, they bind to cell surface receptors of the Frizzled family. Frizzled can translocate the signals to the nucleus and function as a transcriptional activator through intracellular β-catenin, a component downstream of the receptor. Study of the Wnt signaling pathway revealed insights into both embryogenesis and oncogenesis. Thus far, close to 100 Wnt genes were isolated from species ranging from human to the nematode *C. elegans* [Wodarz and Nusse, 1998]. The first Wnt gene, mouse Wnt-1, was discovered in 1982 as a proto-oncogene activated by integration of mouse mammary tumor virus in mammary tumors [Nusse and Varmus, 1982]. Deregulated activation of the Wnt pathway drives cell proliferation by turning on genes encoding oncoproteins and cell-cycle regulators [Peifer and Polakis, 2000]. Recent studies provided evidences that Wnt signaling pathway regulates stem cell self-renewal in ES cells and adult tissues [Huelsken et al., 2001; Kielman et al., 2002; Reya et al., 2003].

One particular interesting pathway that regulates both stem cell self-renewal and tumorgenesis is JAK-STAT signaling pathway, which regulates normal cellular events including differentiation, proliferation, and apoptosis. Abnormal activation of STAT-signaling also gives rise to cell transformation and oncogenesis, such as breast cancer, brain tumors, melanoma, prostate cancer, and leukemia [Calo et al., 2003]. For many cancers, the mechanism of malignant transformation is not well understood. The blockade of lineage differentiation might enforce stem cell self-renewal. The stem/progenitor cells of tissues are the target cells in some cancer, such as certain types of acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL) and chronic myeloid leukemia (CML) [Bonnet and Dick, 1997; George et al., 2001; Mauro and Druker, 2001]. Blockade of stem/progenitor cell differentiation in G1 phase of cell cycle could result in growth indefinitely and accumulation stem/progenitor cells. Pluripotent embryonic cells have rapid cell division with a short G1 phase [Resnick et al., 1992; Savatier et al., 1994; Kranenburg et al., 1995]. Such rapid rate of cell cycle is associated with low expression of D-type cyclins, lack of CDK4 kinase activity, constitutive phosphorylation of pRB and resistance to growth inhibition mediated by $p16^{ink4a}$ [Savatier et al., 1994, 1996], which are common features of cancer cells [Lukas et al., 1995; Medema et al., 1995].

CONCLUSIONS AND FUTURE DIRECTIONS

Although there is significant therapeutic potential of stem cells for treatment of human disease and injury, there are scientific obstacles that need to be overcome. Self-renewal is a common feature of stem cells. The molecular mechanisms that regulate self-renewal might be shared by different types of stem cells, adult stem cells, embryonic stem cells, and cancer stem cells. Adult stem cells exist in many tissues, and are part of a nature system for tissue repair. Therefore, adult stem cells for autologous therapy have great potential because of no immune response and no tendency of malignant. However, most isolated adult stem cells from bone marrow, fat, muscle, and nervous tissue have a limited differentiation potential, and have limited life span in vitro. Understanding of molecular mechanisms governing stem cell self-renewal may help to expand adult stem cells in culture. In contrast, ES cells have potential to differentiate into essentially all cell phenotypes, and can be readily expanded in culture. However, scientific challenges for ES-cell therapy include animal product contamination, tumorigenicity, immunocompatibility, isolation of desired cell types, and availability of suitable animal models to test cell function. For example, when undifferentiated human ES cells are injected subcutaneously, intramuscularly, or into the testis, they form teratocarcinoma-like tumors in adult mice [Thomson et al., 1998; Reubinoff et al., 2000; Odorico et al., 2001]. These tumors contain differentiated cells derived from all three germ layers. Therefore, any ES cell-based therapy has the risk of tumor formation from undiffer-

entiated ES cells. Studies of mouse ES cells indicated that differentiated ES cells are less likely to generate teratomas after transplantation [Werning, 1975; Brustle et al., 1997; Plachta et al., 2004]. Due to spontaneous differentiation in ES cells, differentiated human ES cells contain heterogeneous multiple cell types simultaneously. Therefore, one of the major challenges in stem cell research is to obtain sufficient and desired cell types for clinical applications. Selecting progenitor cells rather than mature cell type from differentiated ES may be advantageous for in vitro expansion and in vivo function. Understanding molecular mechanisms governing ES cell differentiation to a specific cell lineage. Recently, three groups have demonstrated that pluripotent stem cells can be generated from mouse fibroblasts by enforced expression of four transcription factors, including Oct4, Sox2, c-Myc, and KLF4, indicating the fundamental roles of transcription factors in nuclear reprogramming [Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007]. These studies could potentially lead to patient-based stem cell therapies.

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